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Metagenomic analysis of the impact of nitrofurantoin treatment on the human fecal microbiota

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Abstract

Objectives. To study changes in the fecal microbiota of patients with uncomplicated urinary tract infections (UTI) treated with nitrofurantoin, and of non-treated healthy controls using 16S rRNA analysis.

Methods. Serial stool samples were collected from patients receiving nitrofurantoin treatment at different time points; before treatment (day 1; T1), within 48 hours of end of treatment (day 5-15; T2) and 28 days after treatment (day 31-43; T3) along with healthy controls. Direct DNA extraction (PowerSoil DNA Isolation Kit, MoBio Laboratories, Carlsbad, CA, USA) from stool samples was followed by pyrosequencing (454 GS FLX Titanium) of the V3–V5 region of the bacterial 16S rRNA gene.

Results. Among UTI patients, mean proportions of the Actinobacteria phylum, increased by 19.6% in the first follow-up sample (T2) in comparison to the pre-treatment baseline (T1) stool sample ($P = 0.026$). However, proportions of Actinobacteria reversed to 'normal' pre-antibiotic levels, with a mean difference of 1.0% compared to baseline proportions, in the second follow-up sample (T3). The increase in Actinobacteria was specifically due to an increase in the Bifidobacteriaceae family (*Bifidobacterium* genus), which constituted 81.0% (95% CI: ±7.4%) of this phylum.

Conclusions. No significant impact was observed other than a temporary increase in the beneficial *Bifidobacterium* genus following nitrofurantoin treatment, which supports its reintroduction into clinical use.

Introduction

Nitrofurantoin is a synthetic nitrofuran compound that has been used for decades for the effective treatment of lower uncomplicated urinary tract infections (UTIs). [1] Upon oral administration, most of the nitrofurantoin is rapidly absorbed in the small intestine and excreted by the kidneys into urine where it reaches high therapeutic concentrations (200 µg/mL). [2] So far, clinically significant resistance in most uropathogens is uncommon. [3] However, as a small amount (6 to 13%) of orally administered nitrofurantoin also reaches the colon, [1, 4] it might impact the bacterial composition of the gastrointestinal tract. Nitrofurantoin-resistant (NIT-R) strains have been detected previously in the feces of both nitrofurantoin-treated patients and healthy volunteers, although with a very low prevalence (0.6 - 2%). [5] As nitrofurantoin targets important bacterial nitroreductases, [3] this low recovery of NIT-R strains might be ascribed to an associated fitness cost, especially in presence of nitrofurantoin. [5] As part of a prospective, cohort study in ambulatory care, we studied the impact of nitrofurantoin treatment on the the gastrointestinal flora of patients with uncomplicated UTIs (ISRCTN 26797709). An initial screening of these samples on culture detected very low prevalences (1.6 − 3.3%) of NIT-R Enterobacteriaceae in the gastrointestinal flora of nitrofurantoin-treated patients both pre- and post-treatment (A. Stewardson, unpublished data). Furthermore, differences in colony counts of Enterobacteriaceae (susceptible or resistant) either between UTI patients and controls, or between the different time-points of the patient and control groups, were also unremarkable. The stability of the culturable gastrointestinal flora under nitrofurantoin treatment prompted us to investigate in the present study potential changes in the non-cultured fraction utilizing culture-independent screening methods.

Materials and Methods

Study design and sampling

This study was part of a prospective, cohort study carried out in ambulatory patients with uncomplicated UTI visiting general practices in Antwerp (Belgium), and Lodz (Poland) during January 2010 – August 2013. Serial stool samples were collected from patients receiving nitrofurantoin treatment (100 mg, 3x daily, 3-15 days, n = 61) before treatment (day 1; T1), within 48 hours of end of treatment (day 5-15; T2), and 28 days after treatment (day 31-43; T3). In parallel, stool samples were also collected from non-antibiotic treated control patients presenting either with minor trauma or for a gynaecologic exam to the outpatient clinics.. Of these, 5 nitrofurantoin-treated patients and 4 controls were included in the present study. Exclusion criteria for UTI patients were treatment with systemic antibiotics within 2 months or hospitalization within 30 days; residence in a longterm care facility; current urinary catheter; and renal transplant or renal replacement therapy. The controls and household contacts received no antibiotics during the study period or 2 months before it. Approval was granted by the Medical Ethics Committee, University of Antwerp Hospital (B30020109056), and by the Bioethics Committee, Medical University of Lodz (RNN/127/10/KE). Subjects provided informed consent.

DNA extraction and 16SrDNA sequencing

We performed a 16S rDNA metagenomic analysis of 15 stool samples from 5 nitrofurantoin-treated patients and 12 stool samples from 4 controls. Total bacterial DNA was directly extracted by PowerSoil DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA, USA) according to an adapted protocol of the Human Microbiome Project. [6] From each stool sample, 2 grams were homogenized with 5 mL MoBio lysis buffer in a stomacher, followed by centrifugation for 5 min at 1500 gcf. Of the resulting supernatant, 1 mL was transferred to

a MoBio Garnet Bead tube and processed according to manufacturer's protocol. DNA concentration was measured by NanoDrop (Thermo Scientific, Waltham, MA, USA). If the DNA concentration was <10 ng/μL, an additional concentration step was included, consisting of precipitation with 0.1 volume 3M sodium acetate and 2 volumes 100% ethanol followed by a washing step with 70% ethanol. 16S rDNA V3-V5 regions were amplified by PCR using V345_341F (CCT ACG GGR SGC AGC AG) and V345_909R (TTT CAG YCT TGC GRC CGT AC) primers. Purified and quantified PCR products were pooled in equimolar amounts and sequenced on ¼ run with the 454 GS FLX Titanium Sequencer (Roche, Basel, Switzerland) using Titanium FLX reagents, resulting in 3500 reads with a 400-600 bp read length on average per sample (Microsynth, Balgach, Switzerland).

Data analysis

Data were analyzed using the online server, MetaGenome Rapid Annotation using Subsystem Technology (MG-RAST). [7] Raw reads were preprocessed with default parameters and searched against the rRNA database using BLAT for rRNA identification, [8] with a cluster cutoff value of 97% identity. The longest sequence of each cluster was used as a representative for a BLAT similarity search performed against the M5rna database, integrating RDP II, [9] Greengenes, [10] and SILVA [11] (max. e-value cutoff = 1×10^{5}; min. % identity cutoff = 97%; min. alignment length cutoff = 15 bp). Mean proportions and 95% CI were used to describe changes in proportions of the 16S rDNA reads assigned to the different phyla present in the stool samples of UTI patients and controls over the three time points. The raw sequence reads generated from this study were deposited to European Nucleotide Archive Accession number PRJEB8375. The effect of nitrofurantoin use on mean proportions of the different phyla (based on 16S rDNA reads) between and within study groups for different time points was statisticalaly analyzed in a generalized linear mixed model in SAS (version 9.4). [12]

Results

At baseline (T1), the three most highly represented phyla in the patient group were the Firmicutes, Verrucomicrobia and Proteobacteria with mean proportions of 56.9% (95% CI: ±21.6), 18.6% (95% CI: ±31.8) and 11.2% (95% CI: ±12.1), respectively. In the control group, these were the Firmicutes, Actinobacteria and Verrucomicrobia with mean proportions of 46.0% (95% CI: ±16.6), 21.9% (95% CI: ±15.1) and 15.1% (95% CI: ±20.8), respectively. However, large variations in proportions of the different phyla existed between individuals, as is evident from the wide 95% CIs, regardless of study group or time point.

Among UTI patients, mean proportions of Actinobacteria increased by 19.6% in the first follow-up sample (T2) in comparison to the pre-treatment baseline (T1) stool sample (P = 0.026) (Table). However, proportions of Actinobacteria reversed to 'normal' pre-antibiotic levels, with a mean difference of 1.0% compared to baseline proportions, in the second follow-up sample (T3) (Table). Further analysis of the 16S rDNA reads revealed that for four of the five UTI patients, the increase in the Actinobacteria phylum was specifically due to an increase in the Bifidobacteriaceae family, which constituted 81.0% (95% CI: ±7.4%) of this phylum. Furthermore, within Bifidobacteriaceae, all 16S rDNA reads matched the genus *Bifidobacterium*, which are beneficial bacteria commonly used as probiotics in human medicine. [13] Besides Actinobacteria, no other remarkable changes in the fecal microbiota were noted either between or within the patient and control groups. A statistically significant change was observed in the phylum Bacteroidetes between the patient and control groups in the post-treatment T2 samples (Table), however, this change was also observed within the control group between the T1 and T2 samples, indicating that this might be ascribed as a 'normal' variation. Furthermore, mean proportions of Bacteriodetes in the fecal microbiota at the three time-points ranged from 1.6 - 4.6%, and any variations therein were probably too minor to be of clinical relevance.

Of note, we did not utilize the 16S reads to calculate the absolute number of bacteria assigned to each phylum but rather compared absolute numbers of 16S rDNA reads assigned to the different phyla to arrive at mean proportions of the phyla. This was firstly because of the lack of availability of sequencing data, and therefore of the 16S copy numbers, for several phyla or genera in the fecal microbiota, and secondly, because of the reported variations in 16S copy numbers at various taxonomic levels.[14] Furthermore, we utilized 454 sequencing (vis-à-vis Illumina) as it has an advantage in terms of producing longer read lengths covering multiple variable regions, which allows more reliable clustering for species determination.

Discussion

Utilizing a culture-independent approach, the present study did not show any significant impact of nitrofurantoin treatment on the fecal microbiota other than a temporary increase in the Actinobacteria phylum and more specifically in the beneficial *Bifidobacterium* genus. In addition to its low impact on the bacterial composition of the gastrointestinal flora, resistance selection by nitrofurantoin was also minimal, as observed by the low recovery of NIT-R Enterobacteriaceae from urine and stools of treated patients. [5] This is most likely due to the fitness costs, observed as growth deficits, associated with acquisition of NIT-R conferring mutations in the target genes, as shown by us recently. [5] Furthermore, a recent epidemiological study on clinical NIT-R *E. coli* strains noted a lack of clonal spread of such isolates in the community, reiterating the high fitness costs associated with nitrofurantoin resistance in *E. coli* .[15]

However, despite its favourable profile, nitrofurantoin was earmarked as one of the older, potentially useful, but 'forgotten' antibiotics by the ESCMID (European Society of Clinical Microbiology and Infectious Diseases) Study Group for Antibiotic Policies (ESGAP) in 2006. [16] ESGAP's review of literature regarding reasons for disappearance of such antibiotics from clinical use revealed a combination of market failures and failures in production and regulatory processes with non-availability of narrow-spectrum antibacterials forcing clinicians to use broad-spectrum drugs thus adversely influencing prudent antibiotic use policies.[17],[18] Reasons for shortages and market withdrawals of older antibiotics might in turn have been related to lack of profit for drugs in limited market areas (small countries) and increasing regulatory requirements and bureaucracy.[17],[18] However, with the current escalation in antibiotic resistance rates and a lack of new antibiotics, nitrofurantoin's unique mechanism of action, site specificity, achievement of high urinary levels and low serum concentrations, and its effectiveness against both Gram-negative and Gram-positive bacteria provide

many advantages in UTI therapy that many of the newer agents do not.[19] Taken together, these data support the reintroduction of this antibiotic in clinical use and also prompt the development of nitrofurantoin derivatives or even of similarly highly ‘targeted’ antibiotics for other infection sites.

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Transparency declarations

None to declare.

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Phyla	UTI patients (n=8)		Controls (n=5)		UTI patients vs controls	
	Difference in proportion (95% CI)	p	Difference in proportion (95% CI)	p	Difference in proportion (95% CI)	p
Actinobacteria						
T1	-	-	-	-	-16.5% (-35.6 to 2.5)	0.084
T2	19.6% (2.7 to 36.6)	**0.026***	-11.3% (-30.3 to 7.6)	0.221	14.4% (-4.7 to 33.4)	0.128
T3	1.0% (-15.9 to 18.0)	0.899	-14.7% (-33.6 to 4.3)	0.119	-0.8% (-19.9 to 18.2)	0.926
Bacteroidetes						
T1	-	-	-	-	0.1% (-2.2 to 2.3)	0.959
T2	0.5% (-1.3 to 2.2)	0.559	2.8% (0.9 to 4.8)	**0.008***	-2.3% (-4.5 to -0.1)	**0.044***
T3	-0.3% (-2.0 to 1.5)	0.740	1.0% (-0.9 to 3.0)	0.271	-1.3% (-3.5 to 1.0)	0.245
Firmicutes						
T1	-	-	-	-	10.9% (-19.8 to 41.6)	0.457
T2	-9.1% (-29.9 to 11.7)	0.365	2.0% (-21.3 to 25.3)	0.856	-0.2% (-30.9 to 30.5)	0.992
T3	-1.7% (-22.6 to 19.1)	0.862	8.5% (-14.8 to 31.8)	0.448	0.7% (-30.0 to 31.4)	0.960
Proteobacteria						
T1	-	-	-	-	8.2% (-1.0 to 17.3)	0.077
T2	-5.8% (-14.4 to 2.9)	0.175	-1.2% (-10.9 to 8.5)	0.795	3.6% (-5.6 to 12.8)	0.413
T3	-2.8% (-11.5 to 5.8)	0.496	0.2% (-9.5 to 9.9)	0.968	5.2% (-4.0 to 14.3)	0.247
Tenericutes						
T1	-	-	-	-	-2.1% (-5.2 to 1.0)	0.166
T2	0.2% (-2.3 to 2.7)	0.871	-1.5% (-4.1 to 1.1)	0.230	-0.4% (-3.6 to 2.8)	0.786
T3	2.4% (-0.4 to 5.1)	0.083	0.7% (-1.9 to 3.3)	0.553	-0.4% (-3.8 to 3.0)	0.801
Verrucomicrobia						
T1	-	-	-	-	0.9% (-34.2 to 36.1)	0.954
T2	-9.4% (-35.8 to 17.0)	0.447	8.8% (-13.2 to 30.8)	0.392	-17.3% (-54.2 to 19.7)	0.322
T3	4.8% (-18.9 to 28.4)	0.664	6.1% (-15.8 to 28.1)	0.548	-0.4% (-35.7 to 34.9)	0.979
Unclassified						
T1	-	-	-	-	-0.3% (-7.4 to 6.9)	0.936
T2	1.2% (-3.1 to 5.4)	0.557	0.3% (-4.5 to 5.1)	0.893	0.6% (-6.5 to 7.8)	0.856
T3	-1.2% (-5.5 to 3.1)	0.556	-1.9% (-6.6 to 2.9)	0.416	0.4% (-6.8 to 7.5)	0.909

Table: Changes in mean proportions of 16S rDNA reads assigned to different phyla from baseline and between study groups. *: Statistically significant difference with $p<0.05$, generalized linear mixed model. T1, day 1; T2, day 5-15; T3, day 31-43.